Asian Herpetological Research 2012, 3(2): 151–156
DOI: 10.3724/SP.J.1245.2012.00151

Effect of Dietary Vitamin C on the Antioxidant Defense System of Hibernating Juvenile Three-keeled Pond Turtles (*Chinemys reevesii*)

Qi ZHANG, Cuijuan NIU* and Weijie XU

Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, *College of Life Sciences*, *Beijing Normal University*, *Beijing*, 100875, *China*

Abstract Juvenile three-keeled pond turtles (*Chinemys reevesii*) were fed diets supplemented with vitamin C (Vc) at doses of 0 (basal diet, Vc0), 100 (Vc100), 200 (Vc200), 500 (Vc500) and 2500 (Vc2500) mg/kg diets at 28°C for 4 weeks, respectively. Then, the water temperature was gradually reduced to 10°C, and the turtles were induced into hibernation. Liver tissue samples were collected at three time points: start of hibernation (T1), 4 and 6 weeks' hibernation (T2 and T3). A control group fed with the basal diet was set to parallel the whole treatment process, but reared at 28°C constantly. The results showed that hibernation mildly affected the antioxidant system and the influence varied with hibernating time. Hepatic malondialdehyde content of the Vc100 group was significantly lower than that of the other groups at T1. At T2, hepatic MDA in the groups of Vc500 and Vc2500 decreased significantly, while no clear differences were found among all groups at T3. The activities of antioxidant enzymes showed a positive correlation with dietary Vc dose before hibernation. After hibernation, total antioxidant capability was not affected by Vc. Superoxide dismutase activity became similar in different groups at T2, but decreased in higher Vc groups (≥ 200 mg/kg) at T3. Glutathione peroxidase and glutathione-S-transferase activities decreased significantly with dietary Vc supplementation (≥ 100 mg/kg) at T2, but recovered at T3. The result indicates that under normal rearing condition, low dietary Vc supplementation (< 100 mg/kg) might be beneficial to the antioxidant defense system. The effect of dietary Vc on the antioxidant defense system differed during hibernation.

Keywords *Chinemys reevesii*, hibernation, vitamin C, antioxidant capability

1. Introduction

The three-keeled pond turtle (*Chinemys reevesii*) is a fresh water turtle species with great economic value. Turtle breeding in China has become a developing industry (Zhang, 2000). However, one of the big problems in turtle breeding is the high mortality of juvenile turtles soon after hibernation. In nature, turtles go into hibernation when the temperature is low (Reese *et al*., 2001). Reactive oxygen species, generated during hibernation especially when the environment is anoxic (Duranteau *et al*., 1998), will mediate lipid peroxidation and DNA damage (Halliwell *et al*., 2007; Niki, 2009). A well-developed antioxidant defense system can minimizes oxidative damage during hibernation (Baker *et al*., 2007).

Numerous studies have demonstrated that Vitamin C (Vc) has an important role in improving the anti-stress capability and immune function in ectotherms (Dunier *et al*., 1995; Verlhac *et al*., 1998; Zhou *et al*., 2003, 2005). Vc is a well-known antioxidant which protects cell membrane from reactive oxygen species (Hong *et al*., 2002; Liu *et al*., 1998; Ochiai *et al*., 2006). However, there are few reports on the role of Vc during turtles' hibernation (Qian *et al*., 2008). In this study, we evaluated the effect of dietary Vc on the antioxidant defense system of hibernating juvenile turtles to provide basic data for turtle breeding.

2. Materials and Methods

2.1 Animals Juvenile three-keeled pond turtles were reared in 68 × 40 × 38 cm plastic tanks with 8 turtles in

* Corresponding author: Prof. Cuijuan NIU, from College of Life Sciences, Beijing Normal University, with her research focusing on physiological ecology, nutrition and stress responses of aquatic animals, especially fresh water turtles and molecular ecology of zooplankton.
E-mail: cjniu@bnu.edu.cn
Received: 14 November 2011 Accepted: 15 May 2012

each tank. The water depth was kept 6 cm, and water temperature at 28 ± 1°C, with a fixed photoperiod of 10 L: 14 D. Aerated tap water was used with dissolved oxygen more than 5 mg/L at pH 7.9. Turtles were fed to satiety at 03:00 pm everyday, and the diet and feces were removed half an hour later. We changed the water one third once a day with isothermal aerated tap water during the feeding period. The Vc source for the experiment was Vc phosphate (containing 35% Vc) provided by Beijing Sangpu Biological Science and Technology Ltd. Table 1 shows the biochemical composition and energy of the basal diet. We added Vc phosphate in the basal diet to prepare experimental diets which contained Vc of 100 mg/kg, 200 mg/kg, 500 mg/kg and 2500 mg/kg, and the diets were stored in the dark at -20℃. The basal diet was given to the turtles for three weeks before the experiment started.

Table 1 Biochemical composition of the basal diet (Mean ± S. E.).

Moisture (%)	Crude protein (%)	Crude lipid (%)	Crude ash (%)	Energy content (J/mg)
6.33 ± 0.04	42.41 ± 0.53	9.05 ± 0.13	13.42 ± 0.08	18.06 ± 0.09

Healthy juvenile turtles (88.9 ± 2.45 g) were randomly selected and divided into 6 groups: control (basal diet, no hibernation), Vc0 (0 mg/kg), Vc100 (100 mg/kg), Vc200 (200 mg/kg), Vc500 (500 mg/kg), and Vc2500 (2500 mg/kg). Each group contained 3 parallel groups, with 8 turtles in each parallel group. The experimental turtles in the Vc0 to Vc2500 groups were fed with the corresponding diets at 28°C for 4 weeks. Then, the water temperature was gradually reduced to 10°C within one week, thereby inducing hibernation. Liver tissue samples were collected at three time points: start of hibernation (T1, right before the temperature was reduced), and 4 and 6 weeks into hibernation (T2 and T3). The water temperature was kept at 10 ± 1°C during the hibernation, while that of the control group was kept at 28 ± 1°C constantly. Furthermore, the control turtles were fed with basal diet until the end of the experiment. After 48 hours starvation, the turtles were sacrificed rapidly at every sampling time and dissected immediately on ice. The liver tissue samples were collected and stored at -80°C.

2.2 Indexes and analysis The protein and malondialdehyde (MDA) content, total antioxidant capability (T-AOC), superoxide dismutase (SOD), Glutathione peroxidase (GPX) and glutathione-S-transferase (GST) activities were measured with reagent kits provided by the Nanjing Jiancheng Institute of Biological Products according to their instructions. Data, expressed as Mean ± S. E., were statistically analyzed using SPSS 13.0. All data were initially tested by a One-sample Kolmogorov-Smirnov Test and Test of Homogeneity of Variances. The interaction of the two factors (hibernation and rearing time) was checked by two-way ANOVA. Then, Independent-Samples *T*-Test was used to assess the effect of hibernation by comparing the Vc0 group with the control group, while one-way ANOVA was employed to check the changes in different sampling time within the Vc0 group and the control group, respectively. Comparisons were done with one-way ANOVA to check the effect of dietary Vc. If a significant difference was found, least significant difference (LSD) was followed for multiple comparisons. The level of significance was set at $P < 0.05$.

3. Results

3.1 Effects of hibernation and rearing time on the antioxidant defense system The effects of hibernation and rearing time on the antioxidant defense system are shown in Table 2. There were interactions between hibernation and rearing time for all the indexes (two-way ANOVA, $P < 0.05$). No significant differences were found between the control group and the Vc0 group at T2 for MDA content, T-AOC, GPX and GST activities (Independent-Samples *T*-Test, $P > 0.05$). Only the SOD activity of the Vc0 group at T2 was significantly higher than that of the control group ($P = 0.006$). At T3, there were no significant differences between the control group and the Vc0 group for all the indexes (Independent-

Table 2 Effects of hibernation and rearing time on the antioxidant defense system in *C. reevesii* (Mean ± S. E.).

		Control	Vc 0
MDA	T1	1.56 ± 0.22	1.65 ± 0.14^{b}
n = 8	T2	1.50 ± 0.06	1.50 ± 0.05^{b}
	T3	1.84 ± 0.08	1.93 ± 0.06^{a}
T-AOC	T1	0.95 ± 0.06	0.83 ± 0.04
n = 8	T2	0.85 ± 0.07	1.13 ± 0.13
	T3	0.96 ± 0.04	1.07 ± 0.09
SOD	T1	120.23 ± 5.66	96.37 ± 5.03^{b}
n = 8	T2	106.25 ± 4.41	122.79 ± 2.53^{*a}
	T3	115.77 ± 4.50	119.22 ± 4.39^{a}
GPX	T1	8.28 ± 0.38	7.47 ± 0.54^{b}
n = 8	T2	8.92 ± 0.66	9.20 ± 0.34^{a}
	T3	7.08 ± 0.67	7.23 ± 0.40^{b}
GST	T1	47.38 ± 3.01^{b}	41.86 ± 2.23^{b}
n = 8	T2	57.19 ± 2.38^{a}	62.60 ± 1.59^{a}
	T3	42.05 ± 1.35^{b}	39.86 ± 1.27^{b}

Data with different superscript letters are statistically different (differences among T1, T2 and T3 in the same group).
*: indicates statistically significant differences between two groups at the same time ($P < 0.05$).

Samples *T*-Test, $P > 0.05$). For the control group, no significant changes were found through the whole rearing process for MDA content, T-AOC, SOD and GPX activities (ANOVA, $P > 0.05$). The GST activity at T2 was significantly higher than that at T1 and T3 ($P = 0.001$). For the Vc0 group, hepatic MDA, SOD, GPX and GST activities varied clearly at different sampling times (ANOVA, $P < 0.05$), except T-AOC , which did not change much during the experimental trial ($P > 0.05$).

3.2 Effect of dietary Vc on the antioxidant defense system The effect of dietary Vc on hepatic MDA content at different times is shown in Figure 1. At T1, significant differences were found among groups ($F_{4, 33} = 4.722$, $P = 0.004$). The MDA content of the Vc100 group was significantly lower than that of other groups, while there were no clear differences among the other groups. Dietary Vc also showed significant effect on MDA at T2 ($F_{4, 33} = 5.975$, $P = 0.001$). The MDA content was clearly lower in the higher Vc dose groups (Vc500 and 2500). At T3, hepatic MDA became similar among groups ($F_{4, 32} = 2.068$, $P = 0.108$).

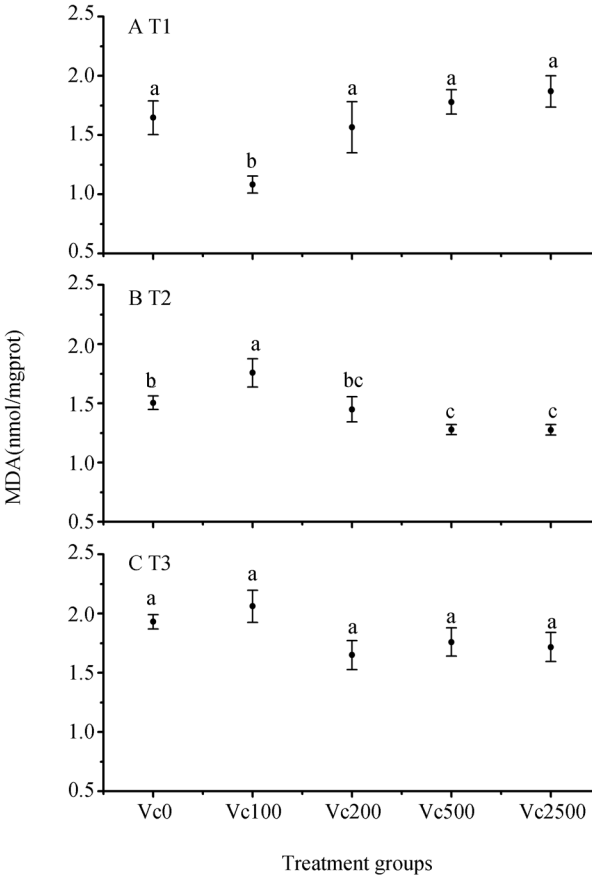

Figure 1 Effect of dietary Vc on hepatic MDA content in *C. reevesii* (Mean ± S. E.). Three sampling time points: Start of hibernation (T1), 4 and 6 weeks' hibernation (T2 and T3). Bars with different superscripts are statistically different ($P < 0.05$). The same below.

As shown in Figure 2, significant differences of hepatic T-AOC at T1 were found among groups ($F_{4, 35} = 5.076$, $P = 0.002$). Dietary Vc was positively related with T-AOC. During hibernation, Vc showed no marked influence on T-AOC (T2: $F_{4, 34} = 1.613$, $P = 0.194$; T3: $F_{4, 35} = 0.672$, $P = 0.616$).

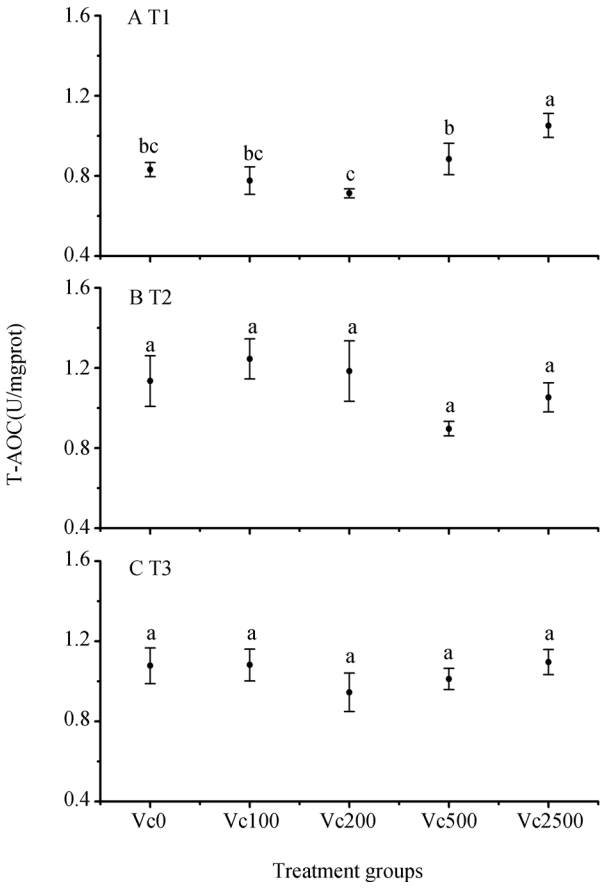

Figure 2 Effect of dietary Vc on hepatic T-AOC in *C. reevesii* (Mean ± S. E.).

Before hibernation, dietary Vc showed a significantly positive influence on the SOD activity (Figure 3 A: $F_{4, 35} = 5.819$, $P = 0.001$). At T2, the SOD activity was similar among groups (Figure 3 B: $F_{4, 34} = 1.988$, $P = 0.118$). At T3, the SOD activity decreased clearly in higher Vc dose groups (≥ 200 mg/kg; Figure 3 C: $F_{4, 35} = 7.959$, $P < 0.001$).

Hepatic GPX activity was not affected by dietary Vc before hibernation (Figure 4 A: $F_{4, 35} = 1.216$, $P = 0.321$). During hibernation at T2, the GPX activity of the dietary Vc supplemented groups was significantly lower than that of the Vc0 group (Figure 4 B: $F_{4, 33} = 4.230$, $P = 0.007$). However, the GPX activity became similar among groups at T3 (Figure 4 C: $F_{4, 35} = 1.536$, $P = 0.213$)

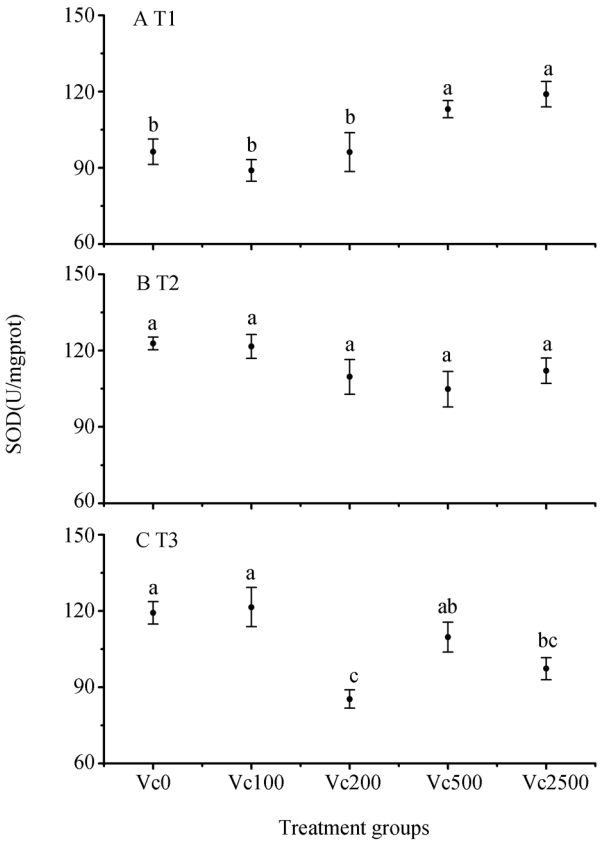

Figure 3 Effect of dietary Vc on hepatic SOD activity in *C. reevesii* (Mean ± S. E.).

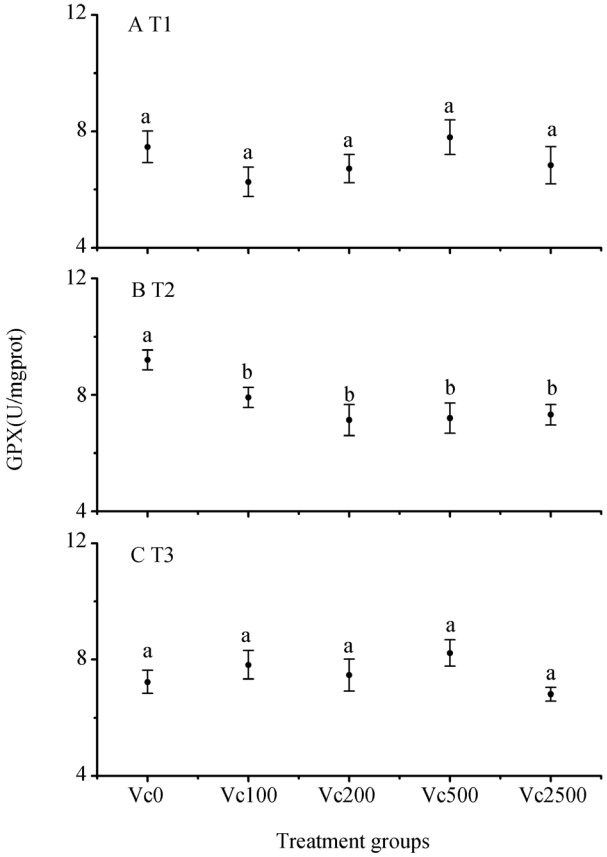

Figure 4 Effect of dietary Vc on hepatic GPX activity in *C. reevesii* (Mean ± S. E.).

Before hibernation, the GST activity of the Vc2500 group was significantly higher than that of the other groups (Figure 5 A: $F_{4, 35} = 3.875$, $P = 0.010$). When hibernated for 4 weeks, the GST activity in higher Vc dose groups (Vc200–2500) became significantly lower than that of the other two groups (Figure 5 B: $F_{4, 34} = 44.081$, $P < 0.001$). At T3, dietary Vc still showed significant influence on the GST activity (Figure 5 C: $F_{4, 34} = 3.659$, $P = 0.013$). The Vc500 group showed the highest GST activity.

4. Discussion

4.1 Effect of hibernation on the antioxidant defense system of juvenile three-keeled pond turtles Hermes-Lima *et al*. (1993) discovered with garter snakes that freezing made muscle CAT, GPX and lung CAT significantly increased. Voituron *et al*. (2006) suggested that the activation of the antioxidant enzyme systems was an effective ecological strategy in response to excessive oxygen free radicals after freezing or arousal. Our results suggested that hibernation showed a mild influence on the antioxidant defense system. Turtles hibernating in normoxic water had only minor disturbances to their acid-base and ionic status, while in anoxic water it became severe (Reese *et al*., 2001). In the context of our experiment, the turtles were hibernated at a water depth of 6 cm at 10°C, which was not extremely cold or anoxic. Biochemical adaptation for natural anoxia tolerance in turtles includes well-developed antioxidant defenses that minimize or prevent damage by reactive oxygen species during hibernation (Willmore *et al.*, 1997). We discovered that hepatic MDA, SOD, GPX and GST activities in the Vc0 group varied clearly at different sampling times (Table 2), which indicates that the influence of hibernation on the antioxidant defense system varies with hibernating time.

4.2 Effect of dietary Vc on the antioxidant defense system of juvenile three-keeled pond turtles In order to make sure that the role of Vc could not be affected by different sampling times, we assessed the role of Vc at time points. Zhou *et al.* (2005) suggested that supplementation of Vc higher than 250 mg/kg was necessary to reduce the adverse effect of acid stress. There are many studies (Mourente *et al*., 2002; Liu *et al*., 2007) reporting the effect of dietary vitamin E supplementation on antioxidant enzyme activities. However, studies on the

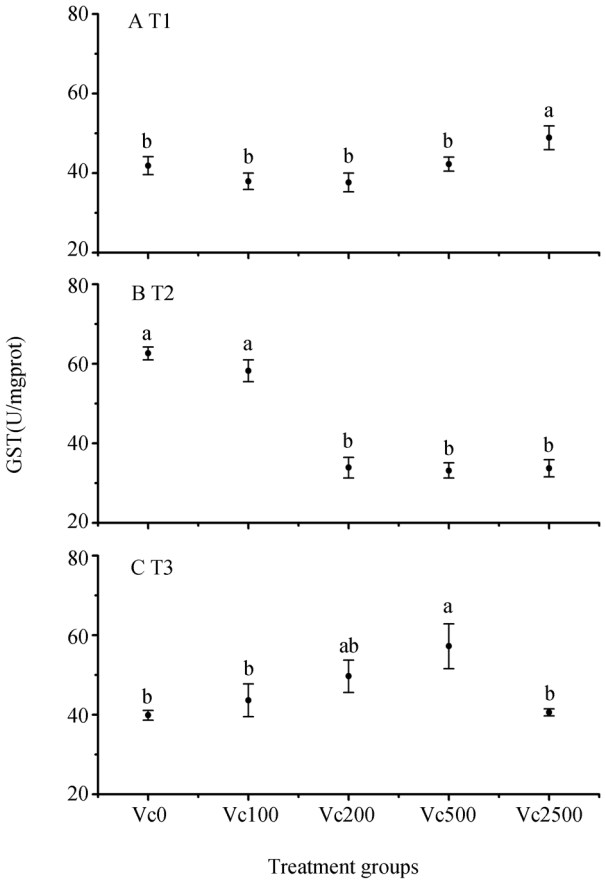

Figure 5 Effect of dietary Vc on hepatic GST activity in *C. reevesii* (Mean ± S. E.).

effect of dietary Vc on an aquatic organism's antioxidant enzyme activities are few. Liu *et al.* (2009) reported that lack of Vc would significantly increase hepatic SOD activity and MDA content in Chinese longsnout catfish. Li *et al.* (2009) reported that the supplementation of Vc could improve antioxidation of Broilers. When dietary Vc increased from 0 to 100 mg/kg and 200 mg/kg, the activity of Cu-Zn SOD was increased by 11.14% and 23.99%, while GPX by 0.48% and 4.95%, respectively. MDA level was decreased by 11.71% when dietary Vc increased from 0 to 200 mg/kg. In our study, the MDA content of the Vc100 group was significantly lower than that of the other groups, while there were no clear differences among the other groups before hibernation (Figure 1 A), indicating that adding a small amount of Vc (Vc100) under normal rearing condition may help to reduce the extent of lipid peroxidation. However, the MDA content did not drop in high Vc dose groups (Vc500, Vc2500); in other words, the positive effect of dietary Vc was not observed. Activities of antioxidant enzymes showed a positive correlation with dietary Vc dose before hibernation (Figures 2, 3, 5 A). Vc may act as an antioxidant, but, simultaneously, it may negatively impact the endogenous protection and repair system (Selman *et al.*, 2006). The result suggests that in high dose of Vc groups (Vc500, Vc2500), although the addition of Vc could improve the vitality of antioxidant enzymes, it simultaneously brings a negative impact. The increased antioxidant capability counteracted the adverse effect brought by high dose of dietary Vc.

Qian (2007) suggested that Vc diet may help protect the liver in juvenile soft-shelled turtles partly due to lipid peroxidation during its hibernation. In our study, the MDA content was clearly lower in the higher Vc dose groups (Vc500 and Vc2500) after four weeks hibernating, but at T3, hepatic MDA became similar among groups (Figure 1 B, C). During hibernation, Vc showed no marked influence on T-AOC. The positive effect of small Vc dose decreased, while high Vc supplementation seemed to play a positive roll in reducing the extent of lipid peroxidation after four weeks of hibernation. The SOD activity at T3, the GPX activity at T2, the GST activity at T2 decreased in higher Vc dose groups, while they changed at other times. We speculated that the requirement of Vc for turtles varied with hibernation time. More investigations should be done for Vc requirements of turtles during hibernation in relation to the hibernating environment.

References

Baker P. J., Costanzo J. P., Lee Jr R. E. 2007. Oxidative stress and antioxidant capacity of a terrestrially hibernating hatchling turtle. J Comp Physiol B, 177: 875–883

Dunier M., Vergnet C., Siwicki A. K., Verlhac V. 1995. Effect of lindane exposure on rainbow trout **(***Oncorhynchus mykiss*) immunity: IV. Prevention of nonspecific and specific immunosuppression by dietary vitamin C (ascorbate-2-polyphosphate). Ecotoxicol Environ Safety, 30(3): 259–268

Duranteau J., Chandel N. S., Kulisz A., Shao Z., Schumacker P. T. 1998. Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. J Biol Chem, 273: 11619–11624

Halliwell B., Gutteridge J. M. C. 2007. Free Radicals in Biology and Medicine, 4th Ed. Oxford: Clarendon Press

Hermes-Lima M., Storey K. B. 1993. Antioxidant defenses in the tolerance of freezing and anoxia by garter snakes. Am J Physiol, 265(3): 646–652

Hong S. Y., Hwang K. Y., Lee E. Y., Eun S. W., Cho S. R., Han C. S., Park Y. H., Chang S. K. 2002. Effect of vitamin C on plasma total antioxidant status in patients with paraquat intoxication. Toxicol Lett, 126: 51–59

Li Y., Yang Z. B., Yang W. R., Zou Y., Zhou J. P., Chu X. N., Wu Y., Mu A. 2009. Effects of supplemental different levels of Vitamin C in diets on antioxidantion and immune function in Broilers. J Shandong Agric Univ (Nat Sci), 40(2): 229–234 (In Chinese)

Liu H. Y., Lei W., Zhu X. M. , Yang Y. X., Han D., Xue M. ,

Xie S. Q. 2009. Effects of different dietary Vitamin C levels on Chinese longsnout catfish (*Leiocassis longirostris* Gtinthe). Acta Hydrobiol Sin, 33(4): 682–689 (In Chinese)

Liu Y., Wang W. N., Wang A. L., Wang J. M., Sun R. Y. 2007. Effects of dietary vitamin E supplementation on antioxidant enzyme activities in *Litopenaeus vannamei* (Boone, 1931) exposed to acute salinity changes. Aquaculture, 265: 351–358

Liu Z. Q., Ma L. P., Liu Z. L. 1998. Making vitamin C lipophilic enhances its protective effect against free radical induced peroxidation of low density lipoprotein. Chem Phys Lipids. 95: 49–57

Mourente G., Dı´az-Salvago E., Bell J. G., Tocher D. R. 2002. Increased activities of hepatic antioxidant defense enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: Attenuation by dietary vitamin E. Aquaculture, 214: 343–361

Niki E. 2009. Lipid peroxidation: Physiological levels and biological dual effects. Free Rad Biol Med, 47: 469–484

Ochiai Y., Kaburagi S., Obayashi K., Ujiie N., Hashimoto S., Okano Y., Masaki H., Ichihashi M., Sakurai H. 2006. A new lipophilic pro-vitamin C, tetra-isopalmitoyl ascorbic acid (VC-IP), prevents UV-induced skin pigmentation through its anti-oxidative properties. J Dermatol Sci, 44: 37–44

Qian M. Y. 2007. Effects of cold stress and dietary Vitamin C on biosynthesis of Vitamin C, non-specific immunity and antioxidant defense in juvenile soft-shelled turtle (*Pelodiscus sinensis*). Master's thesis, College of Life Sciences of Beijing Normal Univ

Qian M. Y., Niu C. J., Jing R. Z., Qian Y. Q., Zhang Z. B. 2008. Effect of dietary Vc and hibernation on biosynthesis of Vc and liver Vc concentration in juvenile soft shelled turtles **(***Pelodiscus sinensis*). Acta Zool Sin, 54(2): 309–316

Reese S. A., Crocker C. E., Carwile M. E., Jackson D. C., Ultsch G. R. 2001. The physiology of hibernation in common map turtles (*Graptemys geographica*). Comp Biochem Physiol, Part A, 130: 331–340

Selman C., McLaren J. S., Meyer C., Duncan J. S., Redman P., Collins A. R., Duthie G. G., Speakman J. R. 2006. Life-long vitamin C supplementation in combination with cold exposure does not affect oxidative damage or lifespan in mice, but decreases expression of antioxidant protection genes. Mech Ageing Dev, 127: 897–904

Verlhac V., Obach A., Gabaudan J., Schuep W., Hole R. 1998. Immunomodulation by dietary vitamin C and glucan in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol, 8(6): 409–424

Voituron Y., Servais S., Romestaing C., Douki T., Barré H. 2006. Oxidative DNA damage and antioxidant defenses in the European common lizard (*Lacerta vivipara*) in supercooled and frozen states. Cryobiology, 52(1): 74–82

Willmore W.G., Storey K. B. 1997. Antioxidant systems and anoxia tolerance in a freshwater turtle *Trachemys scripta elegans*. Mol Cell Biochem, 170: 177–185

Zhang J. 2000. The new technology of rearing turtles in the greenhouse. Beijing: Scientific and Technological Literature Press (In Chinese)

Zhou X. Q., Niu C. J., Sun R. Y. 2005. The effect of vitamin C on stress withstanding capability in the juvenile soft-shelled turtle (*Pelodiscus sinensis*). Aquacult Nutr, 11: 169–174

Zhou X. Q., Xie M. X., Niu C. J., Sun R. Y. 2003a. The effects of dietary vitamin C on growth, liver vitamin C and serum cortisol in stressed and unstressed juvenile soft-shelled turtles (*Pelodiscus sinensis*). Comp Biochem Physiol, Part A, 135(2): 263–270